Abstract

To elevate the physical relevance and translatability of in vitro skin tissue models, it is important to enhance their structural complexity. With the use of 3D bioprinting and proper bioinks, it is possible to modulate the dermal and epidermal architecture and precisely deposit cells and material at desired locations. In this study, a full thickness skin tissue model was bioprinted using the BIO X. The dermis was bioprinted using primary dermal fibroblasts embedded in GelXA SKIN bioink, and the epidermis, containing a high concentration of keratinocytes embedded in ColMA, was deposited on top of the dermis. The skin model was cultured for a total of 14 days, samples were collected on Day 6, when the air-liquid interface culture was initiated, and at the end of the culture on Day 14. Immunofluorescence staining for human collagen type 1, keratin 14, keratin 10 and filaggrin showed that the expression of all markers increased over time. The collagen network was strengthened in the dermis and a clear self-reorganization of the keratinocytes in the epidermis was noted: with a collection of filaggrin toward the outer layers of the epidermis and a sharp increase of keratin 10 among the keratinocytes. These results demonstrate that a robust skin tissue model can be created using 3D bioprinting, validating the suitability of the technology in this field.

Introduction

Skin, being our first and main interface to external environments, is a highly attractive organ for regeneration, and it has been explored heavily by scientists in the past 40 years (Loai, 2019; Tarassoli, 2017). This is partly due to the wide application area of skin tissue models, ranging from drug screening to cosmetic product testing and wound-healing studies, and partly due to the relatively simple composition of skin tissue, which can be described as two main layers with one main cell type per layer. This simple composition means that several 2D models and culture systems have been established in the past. However, these models do not fully recapitulate native skin and lack the spatial organization that 3D models offer (Loai, 2019; Singh, 2020; Vijayavenkataraman, 2016). To increase the physical relevance and enhance the translatability of in vitro results to in vivo conditions, there is an urgent need to modulate skin tissue models in 3D.

Of the different techniques developed to create 3D skin models, the bioprinting fabrication method has shown to be promising in overcoming challenges related to tissue modeling (Pati, 2016; Singh, 2020), giving researchers the ability to precisely model the desired construction and the freedom to incorporate different cells and materials as desired. In 3D bioprinting, it is critical to use a suitable matrix formulation in order to print a stable 3D structure that supports cellular proliferation and migration while ensuring the stability and mechanical integrity of the construct (Tarassoli, 2017; Vijayavenkataraman, 2016).

In 3D bioprinting, both natural and synthetic biomaterials are combined to form printable bioinks. Natural biomaterials have the benefit of mitigating native cues to cells, while synthetic biomaterials have better mechanical properties (Murphy, 2014). Two of the most common natural biomaterials used in 3D bioprinting are gelatin and collagen. Collagen, the most abundant protein in the body, is a key player in the extracellular matrix (ECM) and facilitates excellent cellular communication (Kular, 2014). Pure collagen, however, is tricky to work with since it has to be kept at 4°C or acidic to avoid solidification.
Gelatin, being derived from collagen, maintains some of the cellular features of collagen but has the chemical benefits of being solid at room temperature and liquid at body temperature. Methacrylated gelatin (GelMA) is a printable bioink at 27°C that can be crosslinked using light, which induces the formation of covalent bonds between the methacrylate groups. The same feature of crosslinkability with methacrylate groups can be added to collagen as well; however, methacrylated collagen (ColMA) still needs to be handled at 4°C to avoid solidifying. The GelXA bioink used in this study is a GelMA-based bioink formulation developed at CELLINK that has been designed to widen the printability window of GelMA and allow for easy formation of complex 3D structures.

To read more about the features of different biomaterials and bioinks, please take a look at our bioink selection guide brochure or visit our webpage.

In this study, we aimed to bioprint a full thickness skin tissue model with a dermis and an epidermis using the BIO X bioprinter, the GelXA SKIN bioink for the dermis and the ColMA bioink for the epidermis. With GelXA SKIN, the dermal 3D structure is robustly bioprinted while the inherent properties of ColMA allow for the formation of a thin, rich epidermal layer. In the dermis, the focus was on evaluating the ECM production, with specific interest in the collagen type 1 production since it is the main protein of the native dermis.

For the epidermis, three differentiation markers were chosen: keratin 14 (K14) for proliferative keratinocytes, keratin 10 (K10) for differentiating keratinocytes and filaggrin as a marker for the cornified layer. The epidermis of native skin has a defined differentiation process in which proliferative keratinocytes attached to the dermis become corneocytes (cornified keratinocytes) in the outermost layers of the skin (Eckhart, 2013). In the different layers of this cornification process, specific differentiation markers are expressed (Figure 1). To assess the development of the epidermis, we chose to evaluate the expression of the indicated markers.

### Materials and methods

#### Cell preparation

Two cell types were used in the skin tissue model: juvenile Normal Human Dermal Fibroblasts (NHDF, C-12300, PromoCell) and juvenile Normal Human Epidermal Keratinocytes (NHEK, C-1-12001, PromoCell). The cells were expanded in 2D prior to being bioprinted according to PromoCell’s expansion protocols (NHDF, NHEK) and used in passage 6-7 (NHDF) and passage 3 (NHEK), respectively. NHDFs were cultured in Fibroblast Growth Medium (C-23010, PromoCell) supplemented with Growth Medium SupplementMix (C-39315, PromoCell), whereas the NHEK were cultured in a development formulation of Keratinocyte Growth Medium from PromoCell with Medium SupplementMix (C-97294, PromoCell); both media were supplemented with 1% Antibiotic-Antimycotic (Gibco, 100x).

#### Bioprinting of skin tissue model

The skin tissue model was designed as a dermal basket, with a solid bottom layer topped with two grid layers and a 6-layer high brim (Figure 2). This was to facilitate entrapment of the post-seeded epidermis. The dermal baskets were bioprinted with GelXA SKIN (CELLINK, Ref #IK3X21110301). In short, 5.7 x 10^6 NHDF was collected, spun and reconstituted to a 250 µL cell suspension, which was then carefully blended into 2.5 mL GelXA SKIN, prewarmed to 37°C, and transferred to an amber cartridge (CELLINK, Ref #CSO010311502). To remove entrapped air bubbles, the cartridge of GelXA SKIN with embedded NHDF was centrifuged 1 minute at 460G. The cartridge was then mounted in a Temperature-controlled Printhead (CELLINK, Ref #00000020346) set at 24°C for 10 minutes in the prepared BIO X bioprinter before starting the print session.
This step was performed to equilibrate the temperature of the GelXA bioink to 24°C, which is the suitable bioprinting temperature for GelXA bioinks for complex structures.

The dermal baskets were then bioprinted at 24°C with the Temperature-controlled Printhead in a 12-well plate at a speed of 4 to 5 mm/s, a pressure of 20 to 40 kPa and the print bed of the BIO X set to 14°C. The 12 replicates of the dermal baskets were then first photocured for 15 seconds (per construct) with the 405 nm photocuring module at a distance of 5 cm above the construct before being submerged in crosslinking agent (CELLINK, Ref #CL1010006001) and supplemented with 10 U/mL thrombin for 5 minutes. The dermal baskets were then washed once with Fibroblast Growth Medium and incubated (37°C, 5% CO₂) for 1 hour with Fibroblast Growth Medium prior to addition of the epidermis.

Addition of epidermis

For the epidermis, a mixture of ColMA (CELLINK, Ref #IK4501022001) and NHEK was used. In short, 12 x 10⁶ NHEK, 1 x 10⁶ per dermal basket were reconstituted to a 240 µL cell suspension and mixed with 240 µL ColMA (8 mg/mL, final concentration 4 mg/mL). The reconstituted ColMA was kept on ice until it was added to the dermal constructs. The fibroblast medium was removed from the dermal baskets and 40 µL of the epidermal bioink-cell mix were added on top of each dermal construct. The constructs were left on the benchtop for 15 minutes to settle before being photocured for 30 seconds (405 nm, 5 cm distance from construct). The samples were then centrifuged for 10 seconds at 10G and photocured for an additional 30 seconds. This was to enhance the contact between the epidermis and the dermis. The skin tissue models were then submerged in the keratinocyte growth medium and incubated (37°C, 5% CO₂) for 6 days before being transferred to an air-liquid interface culture. Air-liquid interface was established by transferring the constructs into transwell inserts supplemented with a suitable volume of keratinocyte growth medium (Figure 3). Medium was refreshed every 2nd to 3rd day.

Analysis

Samples were collected at Day 6 (initiation of air-liquid interface) and at Day 14 (conclusion of experiment) and fixed for histology staining in 4% PFA, according to CELLINK’s Fixation Protocol. Samples were then embedded in paraffin and sectioned following CELLINK’s protocols. They were stained for human collagen type 1 (Atlas Antibodies, Ref #HPA011795, not reported to detect rat-derived ColMA), keratin 10 (Atlas Antibodies, Ref #HPA012014), keratin 14 (Abcam, Ref #ab7800) and filaggrin (Atlas Antibodies,
Ref #HPA030188), following CELLINK’s immunofluorescence-staining protocol and using Alexa Fluor 488 (ThermoFisher, Ref #A-27034 for rabbit and Ref #A-11029 for mouse) as a secondary antibody. The samples were also stained with H&E staining following CELLINK’s H&E staining protocol. All protocols can be found on CELLINK’s webpage under the support tab.

Results and discussion

The fabrication method used for this skin tissue model created an intact and robust construct that maintained its shape throughout the experiment. The H&E staining of sample cross sections initially showed that the connection between the two compartments, the dermis and epidermis, was weak. But at Day 14, the two layers had merged (Figure 4). At Day 14, the epidermis could be seen to smoothly follow the contour of the dermis and the keratinocytes had started to reorganize.

![Figure 4. H&E staining of the skin tissue model at Day 6 and Day 14, at 4x and 10x magnification, respectively. Epidermis located to the right in Day 6 images and to the top-right in Day 14 images. Scalebar 200 µm for 4x magnification and 100 µm for 10x magnification.](image)

The intense staining of the dermis in the H&E images makes it hard to separate the fibroblasts from the bioink matrix, but from the immunofluorescence (IF) staining, a clear collagen network formation could be noted (Figure 5). The collagen expression was present already at Day 6 but then extended to Day 14. The number of fibroblasts was also increasing at Day 14, suggesting a proliferation of the fibroblasts within the dermis. The samples were also stained for elastin, but there were no signs of elastin expression, indicating that the conditions in this experiment favor the epidermal formation. Elastin could have been detected in the model if maturated for an extended culture time.

Having a closer look at the epidermal development, the immunofluorescence images showed that the keratin 14 expression was maintained throughout the culture, whereas both the keratin 10 and the filaggrin expression increased on Day 14. Keratin 10, being the marker of differentiating keratinocytes, is expected to be located in the middle section of the epidermis, while filaggrin, a marker for the cornified layer, should be located at the outermost part of the epidermis. The clear increase in keratin 10 and filaggrin expression indicates that the keratinocytes had started to differentiate. The relocation of the filaggrin expression at Day 14 to the top of the constructs, toward the air-liquid interface, shows the cells ability to reorganize within the bioprinted model.
Over the 14-day culture period, the constructs were kept in submerged culture for the first 6 days prior to being transferred to the air-liquid interface culture for 8 days. The bioprinted skin tissue model showed an increased production of collagen in the dermis and reorganized epidermis, where the traditional, stratified differentiation pattern started to form. The stable keratin 14 expression, indicating the presence of proliferating keratinocytes, showed that it would be possible to extend the air-liquid interface culture of the model to further mature the bioprinted skin construct.

Conclusions

This study exemplifies how a full thickness skin tissue model can be 3D bioprinted using a primary cell culture system and CELLINK’s 3D bioprinting platform.

- The cell culture system of cells and mediums from PromoCell enables creation of full thickness skin tissue models. In the current model, the cells reorganized and proliferated to form a more native cellular structure.
- The GelXA SKIN bioink provided a favorable environment for dermal development and the ColMA-based epidermal bioink supported the epidermal formation within the skin tissue model.
- The model design formed a robust platform for both epidermal and dermal development, remaining stable over a 14-day culture period, but it could be cultured for longer to allow further maturation of other dermal and epidermal markers.

References
